be higher than that was observed, this method should still give a reliable indication of relative enzyme activity.

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Cold-Stable Microtubules from Brain†

Bradford C. Webb and Leslie Wilson*

ABSTRACT: When microtubules are polymerized in crude brain extracts, a significant percentage of the microtubules formed do not depolymerize at 0 °C. These cold-stable microtubules are discarded during usual warm-assembly, cold-disassembly microtubule purification schemes and have received little attention. The yield of cold-stable microtubules formed in crude extracts is a function of the $CaCl_2$ concentration present during initial homogenization. Homogenization of mouse brains in buffer containing 1 mM EGTA resulted in a low yield of cold-stable microtubules (less than 10% of the total microtubule assembly, as determined by viscometry), while initial homogenization in 2 mM $CaCl_2$ increased the yield to 47% or higher. Cold-stable microtubules were purified by two cycles of polymerization and depolymerization using Ca^{2+}, instead of low temperatures, to depolymerize the cold-stable microtubules. The protein composition of the purified cold-stable microtubules was not distinguishable from that of cold-labile microtubules on the basis of gel electrophoresis and isoelectric focusing. The two types of microtubules were also not distinguishable by negative-stain electron microscopy. The cold stabilization of brain microtubules may be determined, at least in part, by a low molecular weight substance whose binding to microtubule protein is sensitive to Ca^{2+}. Our results indicate that cold stability is conferred upon incorporation of a cold-stabilizing factor (CSF) into microtubules during the polymerization process, and the ratio of the cold-stabilizing factor to total tubulin determines the proportion of cold-stable microtubules which form. The cold-stabilizing factor may serve an important role in the control of neuronal microtubule assembly or function.

Microtubules exhibit considerable variation in stability when exposed to low temperatures, high hydrostatic pressures, or drugs that inhibit microtubule polymerization. For example, outer doublet microtubules of sea urchin flagella are very stable and do not depolymerize at 0 °C or in the presence of colchicine, whereas many microtubules found in the cytoplasm of animal cells depolymerize rapidly at 0 °C or in low concentrations of colchicine (Behnke & Forer, 1967; see Snyder & McIntosh, 1976, for a review). Microtubules with different stabilities also coexist within the same cytoplasm. For example, kinetochore microtubules of PtK_1 rat kangaroo fibroblast spindles are stable at 0 °C, whereas interpolar microtubules of the same spindles depolymerize at this temperature (Brinkley & Cartwright, 1975).

Stable outer doublet microtubules from sea urchin sperm tails do not assemble or disassemble during flagellar beating and apparently function by a doublet-doublet sliding mechanism mediated by dynein cross bridges (Satir, 1968; Summers & Gibbons, 1971). By contrast, spindle microtubules are in a "dynamic equilibrium" with tubulin subunit pools (Inoué & Sato, 1967), and the functions of these microtubules appear to be integrally linked to their assembly and disassembly during the mitotic process (Margolis et al., 1978). Thus, an improved understanding of the chemical bases which underly the stability differences among microtubules might reveal important aspects of microtubule biochemistry which are directly related to their cellular functions.

Variations in the stabilities of microtubules could be due to chemical differences in tubulins. Such differences might be due to distinct tubulin genes responsible for the biosynthesis of unique tubulin molecules or to different forms of post-translational modification of a single tubulin molecule. Also, regulation of microtubule stability could be brought about through interaction of specific molecules with the surface or ends of microtubules. Such interactions could include the simple binding of cofactors to previously assembled microtubules, incorporation of cofactors into microtubules during assembly, or the association of complex enzyme systems with microtubules.

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Recently we have shown that the in vitro assembly and disassembly characteristics of tubulin isolated from stable sea urchin flagella outer doublet microtubules are remarkably similar to those of tubulin from bovine brain (Farrell & Wilson, 1978; Farrell et al., 1979a,b). Our results suggest that the assembly and disassembly properties of tubulin from diverse sources are very strongly conserved and that many of the functional properties of microtubules and their stabilities must be determined, at least in part, by the specific association of other molecules with the tubulin backbone.

In the present study, we have purified and partially characterized the biochemical properties of a subclass of microtubules in mouse brain extracts that resists depolymerization at 0 °C. As initially described by Grisham (1976), such cold-stable microtubules are excluded during the normal temperature-reversible, assembly–disassembly purification procedures commonly used and, therefore, have received little attention. We find that the cold stability is not due to the tubulin backbone but appears to be conferred by factor(s) which become incorporated into the microtubules during the process of assembly. This cold-stabilizing factor (CSF)[1] may be important in the cellular control of microtubule function.

Materials and Methods

Preparation of Mouse Brain Crude Extracts. Crude extracts were prepared by homogenizing whole mouse brains (Adult Swiss Webster mice, Simonsen Laboratories, Gilroy, CA) in 1.25 mL/g (wet weight) of 0.1 M Pipes[1] and 1.0 mM $MgSO_4$ (pH 6.75, PM buffer) containing 2 mM $CaCl_2$ (PMC buffer) with a motor-driven Teflon/glass homogenizer (three passes) at 0–4 °C. Crude homogenates were centrifuged at 40000*g* for 30 min at 4 °C or, alternatively, at 40000*g* for 10 min and then at 100000*g* for 30 min to produce the crude extract.

Preparation of Purified Cold-Labile Bovine Brain Microtubules. Bovine brains were obtained from Bent's Slaughtering and Processing, Los Olivos, CA. Bovine brain microtubule protein was prepared by three cycles of microtubule polymerization and depolymerization, essentially by the procedure of Asnes & Wilson (1979). Purification was carried out in 0.1 M Pipes, 1 mM $MgSO_4$, and 1 mM EGTA (pH 6.75, PME buffer) without glycerol. Following the third polymerization, microtubules were further cleaned by centrifugation through a cushion of 50% sucrose (Margolis & Wilson, 1977). Final pellets were stored in liquid nitrogen until use. The purified microtubules contained 75% tubulin and 25% microtubule associated proteins (MAPs). Pure tubulin was isolated from this mixture by phosphocellulose (Whatman P-11) column chromatography (Weingarten et al., 1975).

Viscometry. Crude extract samples (2.0-mL minimum volume) containing 2.5 mM GTP and a 1 mM excess of free EGTA above the known Ca^{2+} concentration were pipetted into Canon-Ubbelhode semimicro capillary viscometers in a circulating water bath at 30 °C. Viscometry measurements were recorded for 30 min, which included the maximum of the microtubule polymerization curve (e.g., see Figure 1). The viscometers were then immersed in an ice-water slurry for 30 min to depolymerize cold-labile microtubules and subsequently reimmersed in the 30 °C bath to measure the residual viscosity due to cold-stable microtubules. Colchicine (final concentration 10^{-4} M) was added to each sample prior to reimmersion in the 30 °C bath to prevent any further microtubule polymerization, so the subsequent viscosity measurements reflected only the cold-stable microtubules. Finally, $^1/_{100}$ of the sample volume of 1.0 M $CaCl_2$ in PM buffer (final Ca^{2+} concentration 10 mM) was added to each sample, totally depolymerizing all cold-stable microtubules and allowing the measurement of a microtubule-free base line. Viscosity values are expressed as specific viscosity (Olmsted & Borisy, 1973a). Cold-stable microtubules were indistinguishable from cold-labile microtubules by negative-stain electron microscopy, and the protein compositions were not distinguishable by gel electrophoresis (see Results). Thus, we assume that the specific viscosity measurements do not distinguish between cold-stable and cold-labile microtubules.

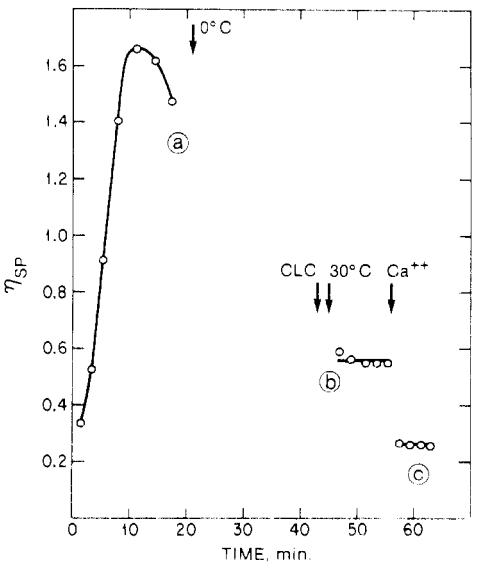

FIGURE 1: Formation of cold-stable microtubules in crude mouse brain extracts. A mouse brain crude extract in PMC buffer, 21.1 mg/mL total protein, was adjusted to 3 mM EGTA and 2.5 mM GTP and polymerized at 30 °C in a viscometer. The arrows indicate the time points at which, sequentially, the viscometer was placed in an ice-water bath, colchicine was added (final concentration 10^{-4} M), the viscometer was replaced in the 30 °C bath, and, finally, Ca^{2+} was added (final concentration 10 mM). The circled letters represent points at which grids were prepared for electron microscopic examination to verify the presence or absence of microtubules and correspond to the micrographs shown in Figure 2a–c.

Purification of Cold-Stable Microtubules. Cold-stable microtubules from mouse brain were purified by two cycles of polymerization and depolymerization using Ca^{2+} and EGTA as selection parameters. Crude extracts (100000*g*) containing 2.5 mM GTP and 1 mM excess free EGTA were polymerized at 30 °C for 15 min. The microtubules were then collected by centrifugation for 30 min at 40000*g* at 30 °C. The pelleted microtubules were Dounce homogenized in PM buffer containing 2–5 mM Ca^{2+} and then centrifuged at 100000*g* for 45 min at 4 °C. The supernatant was adjusted to 1 mM excess free EGTA and 2.5 mM GTP, polymerized at 30 °C for 25 min, and centrifuged through a 50% sucrose cushion in PM buffer in a Beckman SW 50.1 rotor at 200000*g* for 2 h at 30 °C. The pelleted microtubules were gently resuspended with a Pasteur pipet, allowed to cold depolymerize at 0 °C for 30 min, and then centrifuged again at 40000*g* to pellet the cold-stable microtubules. The supernatant from this cold centrifugation was rewarmed to 30 °C for 25 min with added GTP and EGTA and centrifuged warm at 40000*g* for 30 min to collect the cold-labile microtubule pellet.

[1] Abbreviations used in addition to those defined in *Biochemistry* (1966) *5*, 1445: Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; DTT, dithiothreitol; MAPs, microtubule associated proteins; CSF, cold-stabilizing factor; CLC-Tb, colchicine–tubulin dimer complex.

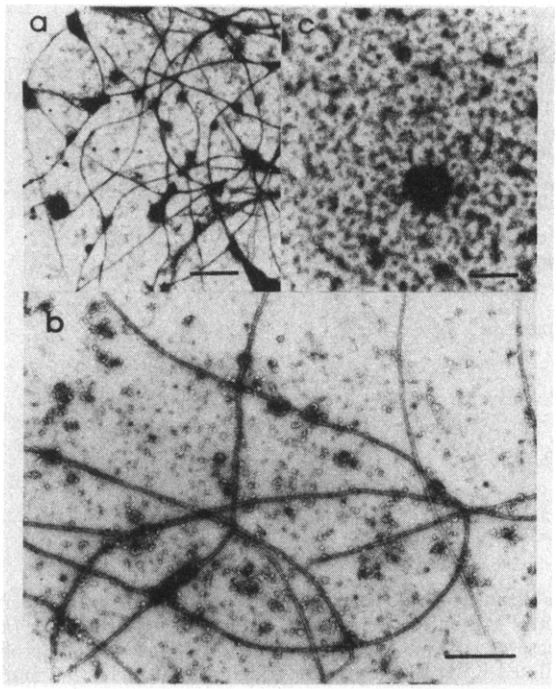

FIGURE 2: Electron micrographs of in vitro polymerized mouse brain microtubules. Electron microscope grids were prepared (see Materials and Methods) during the progress of the experiment shown in Figure 1 to verify the presence or absence of microtubules. Grids were examined in a Philips EM 300 electron microscope, and plates were taken of representative areas on each grid (bar length = 1 μm). (a) Polymerized crude extract; grid prepared after 20 min of polymerization (point a, Figure 1) showing a high density of long microtubules. (b) Cold-stable microtubules; chilling to 0 °C for 25 min did not depolymerize all microtubules (point b, Figure 1). (c) Ca^{2+} treated extract; addition of Ca^{2+} totally depolymerized all of the cold-stable microtubules. No other changes were apparent (point c, Figure 1).

Analytical Methods. The protein constituents of purified microtubules were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis using the methods of Sheir-Neiss (1978) and Weber & Osborn (1969) and also by isoelectric focusing using the method of O'Farrell (1975). Negative-stain electron microscopy was performed as described by Olmsted & Borisy (1973a). Protein concentrations were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Results

Formation of Cold-Stable Microtubules in Crude Brain Extracts. Incubation of 40000*g* supernatant extracts of mouse brain at 30 °C with 2.5 mM GTP in PME buffer resulted in microtubule assembly as determined by viscometry (Figure 1) and electron microscopy (Figure 2a). Cooling the polymerized extract to 0 °C for 25 min resulted in an appreciable but incomplete loss of viscosity which was apparent after rewarming the extract to 30 °C in the presence of 1.0×10^{-4} M colchicine. The addition of colchicine prior to rewarming prevented any new microtubule polymerization without causing significant depolymerization of preexisting microtubules in the short time studied. Much of the residual viscosity was due to the presence of microtubules which did not depolymerize at 0 °C (Figure 2b). These cold-stable microtubules were depolymerized by addition of Ca^{2+} to a final concentration of 10 mM (Figure 2c), which resulted in a decrease of the specific viscosity to a base-line level (Figure 1).

Borisy et al. (1975) have shown that viscosity measurements in crude brain extracts provide an accurate estimate of microtubule mass. Thus, peak viscosity development at 30 °C provides an estimate of total microtubule polymerization, and the residual viscosity after cooling to 0 °C provides an estimate of cold-stable microtubule formation. The percentage of total polymerization due to cold-stable microtubules may be approximated from these viscosity values after subtracting the viscosity value of the Ca^{2+}-induced microtubule-free base line (see Materials and Methods for further discussion). In the experiment shown in Figure 1, this value was 21% of peak microtubule assembly. Similar results were obtained during the polymerization of crude extracts of bovine brain and 13-day-old chick embryo brain (data not shown).

Dependence of Cold-Stable Microtubule Formation on Extraction Conditions. The percentage of cold-stable microtubule formation in 40000*g* mouse brain extracts varied from approximately 5% to as much as 47% depending on extraction conditions. Homogenization of brains in 2 mM Ca^{2+} (PMC buffer) resulted in the largest quantities of cold-stable microtubule formation (20–47% of total) when polymerized with 2 mM excess EGTA, whereas homogenization of brains in 2 mM EGTA (PME buffer) resulted in the lowest quantities of cold-stable microtubules (6–15% of total).

Electron microscopic examination of pellets from brains homogenized in PME buffer after 40000*g* centrifugation revealed the presence of many short microtubule fragments, less than 0.5 μm in length. However, pellets from brains homogenized in the presence of Ca^{2+} (PMC buffer) contained no microtubule fragments. The short fragments may be cold-stable remnants of longer microtubules, present in the brains prior to homogenization, which were fragmented during homogenization. Homogenization in high Ca^{2+} probably depolymerized the cold-stable fragments, thereby augmenting the production of cold-stable microtubules in the subsequent polymerization.

Characteristics of Cold-Stable Microtubule Formation in Crude Extracts. Cold-stable microtubules persisted without depolymerization for at least 4 h at 0 °C. In contrast, cold-labile microtubules depolymerized within 2 min of cooling to 0 °C. Both cold-labile and cold-stable microtubules quickly depolymerized upon addition of 2–5 mM Ca^{2+}. The assembly of cold-stable microtubules was completely prevented by 10^{-4} M colchicine, and griseofulvin at 8.0×10^{-5} M inhibited the assembly of cold-stable microtubules and cold-labile microtubules to the same extent, 75–80%.[2] Cold-stable microtubules formed only under conditions which also supported polymerization of cold-labile microtubules. These included the presence of GTP and elevated temperatures and absence of Ca^{2+} ions. The presence of added ATP, CTP, UTP, GDP, cAMP, cGMP, NADPH, DTT, glutathione, or 2-mercaptoethanol had no discernible effect on the production of cold-stable microtubules. Analysis of microtubule lengths revealed that cold-stable microtubules were 20–36% shorter than the total polymerized microtubule population.

The critical concentration at 30 °C for cold-stable microtubule formation was the same as that for total microtubule formation in crude mouse brain extracts. Extracts were diluted to various total protein concentrations and assayed by viscometry. Total microtubule polymerization and cold-stable microtubule polymerization were determined for each dilution (Figure 3a). A plot of the specific viscosity development for total polymerization and cold-stable microtubule formation vs. protein concentration (Figure 3b) revealed that the critical concentrations were very similar (1.6 mg/mL total protein). Tubulin comprises 22–26% of the total protein, as determined

[2] Early studies (Grisham & Wilson, 1975) indicating that griseofulvin did not prevent polymerization of cold-stable microtubules were carried out at insufficiently high concentrations of griseofulvin.

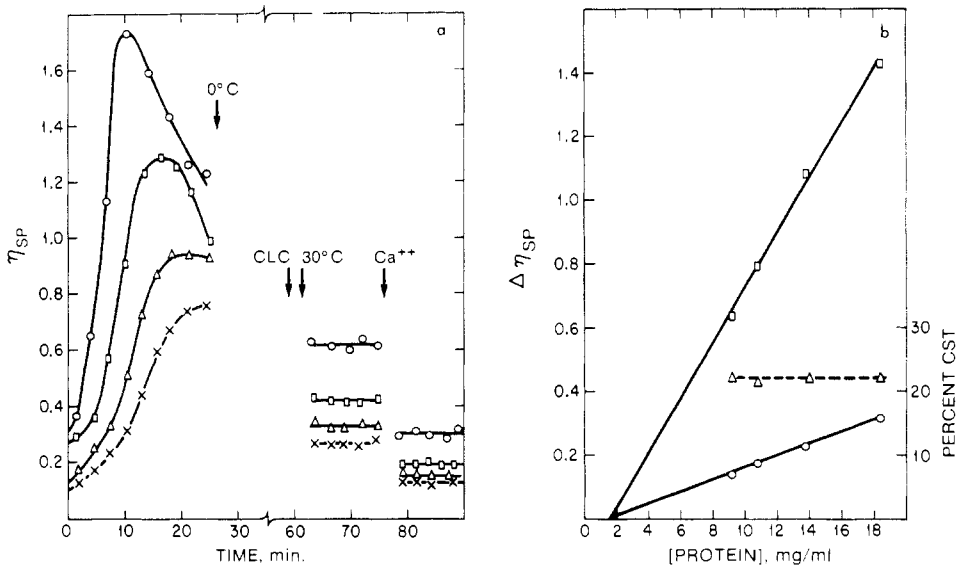

FIGURE 3: Determination of the critical concentration for cold-stable and cold-labile microtubule polymerization. Aliquots of a 40000*g* crude extract adjusted to 2.5 mM GTP and 1 mM excess free EGTA were diluted with PME buffer, 2.5 mM GTP, to crude extract/buffer ratios of 3:1, 1.5:1, and 1:1. The three diluted samples and an aliquot of undiluted extract were assayed by viscometry at 30 °C. (a) Viscosity profiles: undiluted extract, 18.4 mg/mL (circles); diluted 3:1, 13.8 mg/mL (squares); diluted 1.5:1, 10.8 mg/mL (triangles); diluted 1:1, 9.2 mg/mL (crosses). (b) Analysis of viscometry data: total microtubule polymerization (squares) was determined by subtracting the specific viscosity of the Ca^{2+}-induced base-line value from the peak of the polymerization curves for each sample; cold-stable microtubule polymerization (circles) was determined by subtracting the base-line value from the average residual specific viscosity after cooling to 0 °C; percentage of total polymerization as cold-stable microtubules (triangles), right-hand ordinate scale.

by quantitative polyacrylamide gel electrophoresis in Na-DodSO$_4$ (data not shown). Thus, the critical tubulin concentration for both the total microtubule population and the cold-stable microtubule population in the crude extracts was approximately 0.38 mg/mL. The proportion of cold-stable microtubules remained a constant 22% of total microtubule assembly at all dilutions (Figure 3b, dashed line). These results indicate that the sum of the tubulin dimer assembly/disassembly rates at 30 °C for cold-stable microtubules is the same as that for cold-labile microtubules (see Discussion).

Purification of Cold-Stable Microtubules. Cold-stable microtubules are depolymerized in millimolar Ca^{2+}, suggesting that they might be purified using Ca^{2+} and EGTA as selection parameters instead of high and low temperatures. The feasibility of this strategy was verified in preliminary experiments, and the recycling efficiency of cold-stable microtubules was quantitated. A pellet of cold-stable microtubules devoid of cold-labile microtubules was obtained after cold depolymerization and centrifugation of a polymerized crude extract. The microtubules were depolymerized by addition of 3 mM Ca^{2+} in PM buffer. After centrifugation, the resulting supernatant was adjusted to 1 mM excess EGTA and repolymerized at 30 °C with added GTP. By light scattering, approximately 35% of the second cycle microtubules were cold stable.

Cold-stable as well as cold-labile microtubules were purified from mouse brain crude extracts by two cycles of polymerization and depolymerization as shown schematically in Figure 4. A typical purification begun with 15 g of mouse brain produced approximately 80 mg of soluble tubulin in the crude extract. The final yields of purified cold-stable and cold-labile microtubules after the second cycle were 3–5 and 6–8 mg, respectively. The cold-labile microtubules totally depolymerized within 2 min at 0 °C.

Comparison of Cold-Stable and Cold-Labile Microtubules. Negative-Stain Electron Microscopy. The final cold-stable and cold-labile microtubule preparations were pure on the basis of negative-stain electron microscopy. Few aggregates or other identifiable structures (e.g., coated vesicles, polymerized actin)

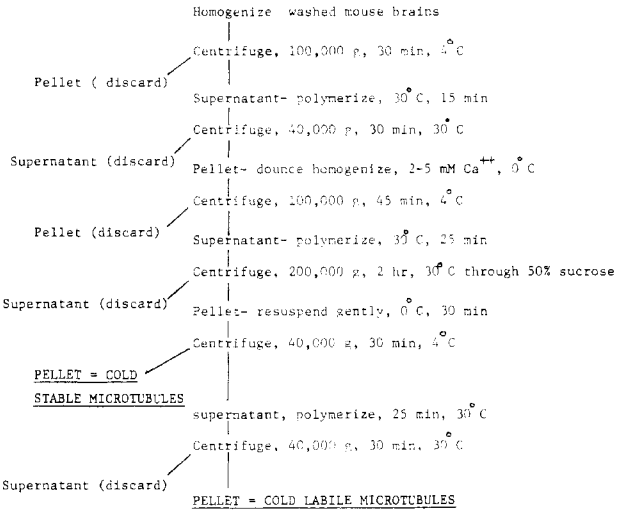

FIGURE 4: Schematic outline of the purification of cold-stable and cold-labile microtubules from mouse brain.

could be detected in the cold-stable microtubule preparation. No cross bridges or other unusual adhering elements were evident (Figure 5a), and the purified cold-stable microtubules could not be distinguished from cold-labile microtubules at high magnification (Figure 5b).

Polyacrylamide Gel Electrophoresis and Isoelectric Focusing. The protein compositions of purified cold-stable and cold-labile microtubules were examined by NaDodSO$_4$–polyacrylamide gel electrophoresis on 6% gels (Figure 6). The protein composition of cold-stable microtubules (gels b, c) was almost identical with that of cold-labile microtubules (gel d) even with heavily overloaded gels (cf. gels c, d). No differences could be detected in the mobility of the tubulin or in the mobility of the high molecular weight microtubule associated proteins. Planimetry of gel scans of cold-stable and cold-labile microtubules revealed that both populations of microtubules

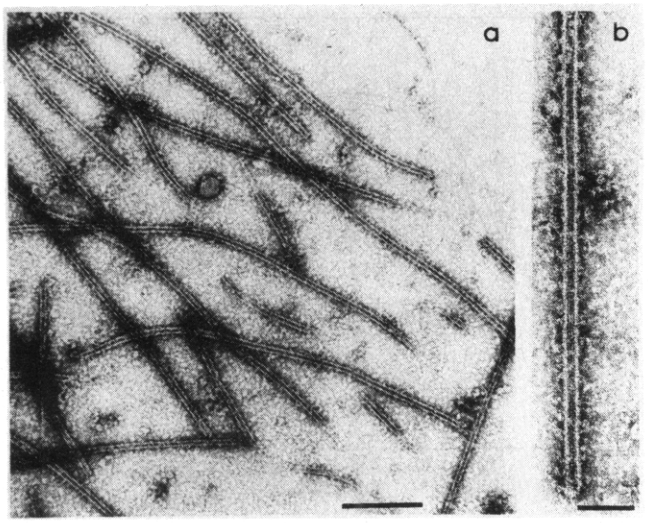

FIGURE 5: Electron micrographs of cold-stable microtubules. Purified cold-stable microtubules, prepared as described under Materials and Methods, were examined by negative-stain electron microscopy with a Philips EM 300 electron microscope. (a) 7200×, bar length = 500 nm; (b) 25200×, bar length = 100 nm.

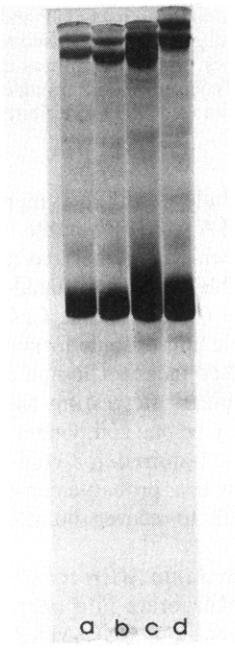

FIGURE 6: Gel electrophoresis in $NaDodSO_4$. Purified cold-stable and cold-labile microtubules, prepared as described under Materials and Methods, were examined by gel electrophoresis on 6% polyacrylamide gels according to the methods of Weber & Osborn (1969) and stained with Fast Green: (a) second cycle microtubule pellet containing 40% cold-stable microtubules and 60% cold-labile microtubules, 56 μg; (b) purified cold-stable microtubules, 38 μg; (c) purified cold-stable microtubules, 95 μg; (d) cold-labile microtubules, 73 μg.

contained 72–75% tubulin and 25–28% high molecular weight microtubule associated protein. Several minor protein bands were evident on gels of both the cold-stable and cold-labile microtubules. These comprise less than 5% of the total protein on the gels and may represent contaminating proteins. Identical results were obtained using the discontinuous $NaDodSO_4$ gel system of Sheir-Neiss (1978) (data not shown).

Tubulin from both cold-stable and cold-labile microtubules could be resolved into seven distinct isoelectric variants on isoelectric focusing gels. However, the cold-stable and cold-labile tubulin patterns were identical (Figure 7, A and B). MAPs were poorly resolved on this gel system and could not be analyzed. Therefore, to the limits of resolution of these

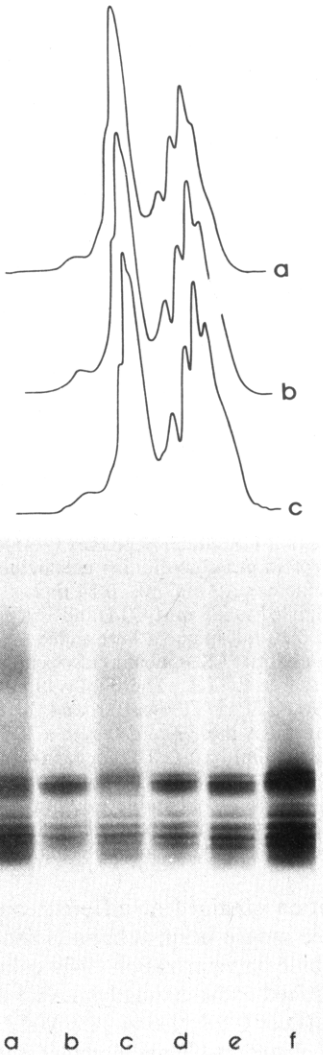

FIGURE 7: Isoelectric focusing. Cold-stable and cold-labile microtubules, prepared as described under Materials and Methods, were analyzed on isoelectric focusing gels according to the method of O'Farrell (1975). (A, top) Gel scans of tubulin region: scan a, cold-labile microtubules, 100 μg; scan b, mixture of cold-labile microtubules (50 μg) and cold-stable microtubules (50 μg); scan c, cold-stable microtubules, 100 μg. (B, bottom) Photographs of the tubulin region: (gels a, b) cold-labile microtubules; (gels c, d) mixture of cold-labile microtubules and cold-stable microtubules; (gels e, f) cold-stable microtubules.

electrophoretic techniques, the protein compositions of cold-stable and cold-labile microtubules were indistinguishable.

Studies on the Mechanism of Cold-Stable Microtubule Formation. Inhibition of Cold-Stable Microtubule Formation with Colchicine–Tubulin Complex from Cold-Labile Microtubules. It was conceivable that the tubulins from cold-stable and cold-labile microtubules, though indistinguishable electrophoretically, were in fact different and that tubulin from cold-labile microtubules could not participate in cold-stable microtubule formation. In one test of this possibility we made use of the fact that colchicine blocks microtubule polymerization substoichiometrically by addition of colchicine–tubulin complexes to microtubule assembly ends, preventing further tubulin addition (Margolis & Wilson, 1977). Colchicine–tubulin complex (CLC-Tb) formed from three-cycle-purified, cold-labile bovine brain tubulin was added in known stoichiometry to aliquots of mouse brain crude extract, and the ability of the CLC-Tb from cold-labile microtubules to inhibit total microtubule formation and cold-stable microtubule formation was determined. A double-reciprocal plot of the

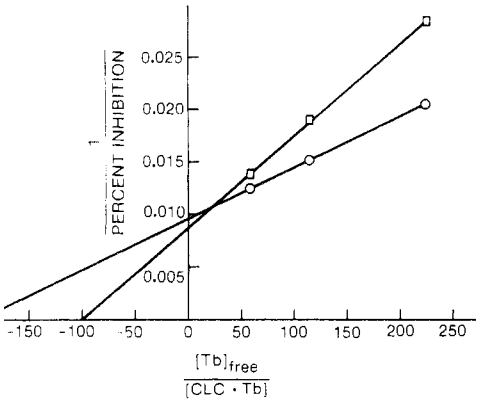

FIGURE 8: Inhibition of polymerization in crude extracts by colchicine–tubulin complex. An aliquot of three-cycle-purified, cold-labile bovine brain microtubule protein was incubated with 10^{-4} M colchicine ([^{3}H]CLC, 9×10^6 cpm/μmol) for 30 min at 30 °C, the incubation mixture was cooled, and the colchicine–dimer complex was isolated by passage through a 1 × 20 cm Sephadex G-25M column at 4 °C. The excluded protein peak, containing colchicine–tubulin complex and no free colchicine (3.9 mg/mL, 0.24 mol of colchicine/mol of tubulin), was adjusted to 2.5 mM GTP and 1 mM EGTA, and 0.0-, 0.05-, 0.10-, and 0.20-mL aliquots were added to 2.0-mL samples of mouse brain crude extract (22% tubulin) also containing 2.5 mM GTP and 1 mM excess free EGTA. The final volumes were adjusted to 2.20 mL with PM/GTP/EGTA buffer, and the four samples were pipetted simultaneously into viscometers in a 30 °C bath for assay. The ratio of (free tubulin)/(CLC-Tb) for each sample was calculated and plotted vs. the reciprocal of the percentage inhibition. Total polymerization, circles. Cold-stable microtubules, squares. The reciprocals of the abscissa intercepts reflect the concentration of CLC-Tb needed for half-maximal inhibition.

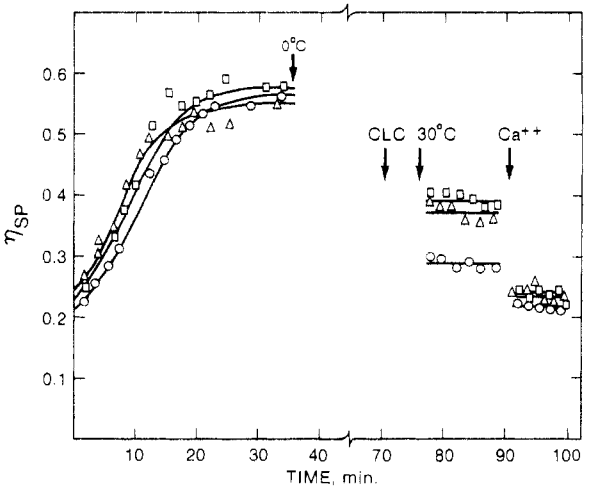

FIGURE 9: Dialysis of crude extracts: the effect of Ca^{2+}. A 100000g mouse brain crude extract was divided into three aliquots after adjusting to 0.1 mM GTP. One aliquot was incubated at 0 °C for 2 h without dialysis to serve as a control, a second aliquot was dialyzed against PMC buffer (250 mL, 2 h, 0 °C), and the third aliquot was dialyzed against PME buffer after adding 1 mM excess EGTA. The samples were then adjusted to 2.5 mM GTP and 1 mM excess EGTA and were pipetted simultaneously into viscometers to measure polymerization competency. Each aliquot was assayed with identical Ca^{2+}, EGTA, and GTP concentrations: triangles, 2 h at 0 °C without dialysis; squares, dialysis vs. PME buffer; circles, dialysis vs. PMC buffer.

percent inhibition obtained at different ratios of CLC-Tb complex to free mouse brain tubulin is shown in Figure 8. Total microtubule polymerization (74% cold-labile microtubules) was inhibited half-maximally at a CLC-Tb complex to free tubulin ratio of 0.5% (1.6×10^{-7} M CLC-Tb), and the polymerization of cold-stable microtubules was half-maximally inhibited at a ratio of 1.0% (3.2×10^{-7} M CLC-Tb). Thus, tubulin from cold-labile microtubules in the form of a CLC-Tb complex must add to the ends of cold-stable microtubules and cold-labile microtubules with equivalent efficacy, providing further evidence that the tubulin in the cold-stable microtubule population is not unique. The results also show that tubulin from bovine brain coassembles with tubulin from mouse brain with approximately similar efficacy.

Evidence for a Low Molecular Weight Cold-Stabilizing Substance. Only cold-labile microtubules form in repolymerized crude brain extracts after removal of the cold-stable microtubules, as determined by electron microscopy and viscometry (data not shown). This indicates that removal of cold-stable microtubules depletes the crude extract of a factor(s) which confers cold stability and that the cold-stabilizing factor(s) (CSF) must be an integral component of cold-stable microtubules.

In our attempts to partially characterize the molecular nature of the CSF, we found that cold stability may be conferred in part by a low molecular weight substance present in the crude extracts which binds to the macromolecular components of microtubules in the absence of Ca^{2+} and is released into solution by millimolar Ca^{2+}. Results of a typical experiment are shown in Figure 9. Aliquots of a crude extract were dialyzed for 2 h at 0 °C in PME or PMC buffer, and the percent cold-stable microtubule formation was compared to an undialyzed aliquot after readjustment to assembly conditions. Forty-five percent of the microtubules which polymerized in the undialyzed sample were cold stable. Similarly, 46% of the microtubules which polymerized in the sample dialyzed against EGTA were cold stable. However, only 20% of the microtubules which polymerized in the Ca^{2+} dialyzed extract were cold stable. In an additional control experiment, incubation for 2 h at 0 °C in 2 mM $CaCl_2$ did not result in any loss of cold-stable microtubule formation. Thus, dialysis in Ca^{2+} for 2 h resulted in a 56% loss in competency to form cold-stable microtubules, suggesting that a low molecular weight substance may be one component, but not necessarily the only component, required for cold-stable microtubule formation. However, as a probable consequence of dilution, it has not been possible to recover the cold-stabilizing activity in the dialysate.

CSF Is Incorporated into Microtubules during Assembly. CSF could either incorporate into microtubules during assembly as a CSF–tubulin or CSF–MAP complex or bind to the surface of already assembled cold-labile microtubules, conferring cold stability. To distinguish between these possibilities, three-cycle-purified bovine brain microtubules were assembly-end blocked, after polymerization had reached steady state, by incubation with 10^{-4} M colchicine for 30 min at 30 °C. The microtubule suspension (total tubulin = 1.3 mg) was then added to a mouse brain extract (total tubulin = 8.0 mg) which had been preincubated with 10^{-4} M colchicine (15 min, 30 °C) to ensure that no microtubule assembly could occur (verified by electron microscopy). The amount of microtubules added was about 20% of the normal polymerization capability of the crude extract. Incubation was continued at 30 °C, and at 10-min intervals samples were removed, cooled to 0 °C, and analyzed by electron microscopy and viscometry for cold-stable microtubule formation. None of the added microtubules became cold stable during 40 min of incubation, indicating that the CSF cannot confer cold stability by binding to the surface of cold-labile microtubules.

Additional evidence that the CSF does not bind to preassembled microtubules but must incorporate into microtubules during assembly is provided by further consideration of the

results of the dilution experiment depicted in Figure 3. In this experiment, the percentage of total polymerization contributed by cold-stable microtubules remained constant over a twofold dilution of the extract. If the CSF bound to tubulin on the surface of preformed microtubules, this would be described by the equilibrium binding equation:

$$K_a = (\text{CLMT})(\text{CSF})/(\text{CSMT})$$

where K_a is the binding constant, (CLMT) is the concentration of tubulin in cold-labile microtubules, (CSMT) is the concentration of tubulin in cold-stable microtubules, and (CSF) is the concentration of the cold-stabilizing factor. Rearrangement of the above equation yields

$$(\text{CSF}) = K_a(\text{CSMT})/(\text{CLMT})$$

This indicates that dilution of the extract would result in a decrease in the proportion of cold-stable microtubules formed since dilution would reduce the CSF concentration. For example, a twofold dilution of the crude extract would reduce the CSF concentration twofold, and the (CSMT)/(CLMT) ratio would also be reduced twofold. In such a dilution experiment (Figure 3) the percentage of cold-stable microtubules remained constant over a twofold dilution, so this mechanism is excluded. We can conclude that the CSF must be incorporated into microtubules during assembly.

Influence of CSF/Tubulin Ratio on Cold-Stable Microtubule Formation. The stoichiometry of CSF incorporation into microtubules, expressed as the CSF/tubulin ratio, should determine the percentage of cold-stable microtubule formation if a specific CSF/tubulin ratio in microtubules is required for cold stability. We have tested this possibility by manipulation of the CSF/tubulin ratio in two ways, first by decreasing and then by increasing the tubulin concentration in crude extracts while leaving the CSF concentration unchanged. In one strategy, tubulin was removed from crude mouse brain extracts by passage of extracts over columns of DEAE-cellulose. The quantity of tubulin removed was determined accurately by polyacrylamide gel electrophoresis. Total polymerization, as determined by viscometry, was strictly proportional to the residual tubulin concentrations (data not shown). In a second approach, three-cycle-purified, cold-labile, bovine brain microtubule protein (75% tubulin, 25% MAPs) was added to aliquots of mouse brain crude extract.

The results of these manipulations are shown in Figure 10a. Unaltered crude extract samples are represented by half-filled symbols. Depletion of tubulin from the crude extracts (unfilled symbols) resulted in a decrease in total polymerization but no decrease in cold-stable microtubule formation until total polymerization was reduced by over 50%. With continued depletion of tubulin, cold-stable microtubule formation decreased. Addition of cold-labile microtubule protein (filled symbols) resulted in an increase in total polymerization and a decrease in yield of cold-stable microtubules. These results indicate that cold-stable microtubule formation was inversely proportional to the total assembly extent, and the percentage of cold-stable microtubules ranged from 64% at the lowest tubulin concentration to 4% at the highest tubulin concentration (Figure 10b). Thus, a specific minimum CSF/tubulin ratio must exist in a microtubule for that microtubule to be cold stable.

Cold-Stable Microtubules Formed with Tubulin from Cold-Labile Microtubules. Crude extracts, depleted of tubulin by DEAE-cellulose chromatography, retain CSF (see above). If this factor rather than a unique type of tubulin is responsible for cold stability, then the addition to the tubulin-depleted extracts of purified tubulin from cold-labile microtubules

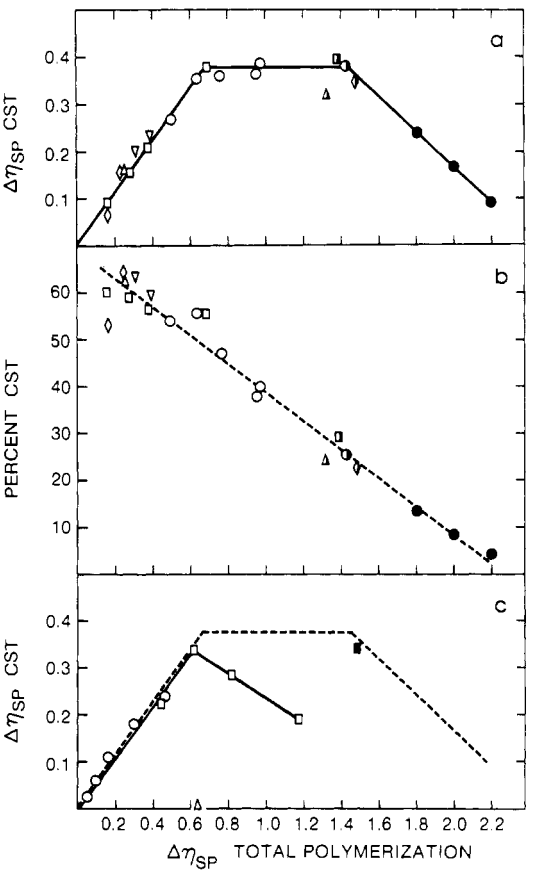

FIGURE 10: Cold-stable microtubule formation: function of tubulin concentration and CSF/tubulin ratio. (a) Cold-stable microtubule formation is plotted as a function of total polymerization extent for crude extract samples which have been augmented (filled symbols) or depleted (unfilled symbols) in tubulin concentration. Unaltered crude extract samples are represented by half-filled symbols. Addition of increasing amounts of three-cycle-purified bovine brain microtubule protein (0.1–0.8 mL, 8.0 mg/mL) to crude extract samples (2.0 mL, 17.8 mg/mL) at constant volume resulted in increased total polymerization and decreased cold-stable microtubule production (filled circles). Aliquots (2.0 mL) of crude extract were passed over 0.5–5.0-cm columns of DEAE-cellulose, preequilibrated in PM buffer, 0.5 mM Ca^{2+}, and 0.1 mM GTP, in Pasteur pipets, to deplete tubulin. Polymerization of the column effluent, after adjusting to polymerization conditions, produced the data points represented by unfilled symbols (five different experiments). (b) The data of Figure 10a are replotted as the percent cold-stable microtubules vs. total polymerization. (c) Conversion of cold-labile tubulin (purified 6S bovine brain cold-labile tubulin) to cold stability was accomplished by addition of 6S tubulin to DEAE-cellulose-treated crude extracts. Addition of tubulin to assembly incompetent (circular symbols) crude extract effluents from 5.0-cm DEAE-cellulose columns increased both total polymerization and cold-stable microtubule formation. The control point (no added tubulin) is the point of lowest total polymerization. The 6S tubulin assayed by itself (with added MAPs) produced no cold-stable microtubules (triangular symbol). In another experiment, addition of 6S tubulin to crude extract samples from 3.0-cm DEAE-cellulose columns produced the profile described by the square symbols. In this experiment, an untreated crude extract sample (filled square) polymerized to the same extent as other untreated samples. The dashed line is a reproduction of the solid line in Figure 10a.

should result in formation of cold-stable microtubules upon polymerization. In such an experiment, purified bovine brain tubulin was added to tubulin-depleted mouse brain crude extracts (Figure 10c). The pure tubulin by itself formed only cold-labile microtubules when polymerized with added MAPs (triangular symbol). However, addition of this tubulin in increasing amounts to aliquots of tubulin-depleted extracts resulted in a concentration-dependent assembly of cold-stable microtubule formation (circular symbols). Beyond a critical

point, further increase in total polymerization resulted in a decrease of cold-stable microtubule formation (square symbols). This behavior qualitatively mimicked the crude extract system (dashed line), but the maximum point of cold-stable microtubule formation occurred at a lower total polymerization extent and did not plateau. We conclude from these results that mouse brain CSF confers cold stability upon microtubules polymerized from tubulin isolated from cold-labile microtubules.

Discussion

The varied resistance of different classes of microtubules to disruption by chemical and physical agents in vitro and in vivo must reflect biochemical differences in tubulins or in specific MAPs. Such biochemical differences may reflect variations in the functional roles of microtubules. Thus, differences in microtubule stability, if understood in chemical terms, could provide insight into some specific biochemical aspects of microtubule function.

Accordingly, the biochemical properties of an unusual cold-stable class of microtubules which polymerizes in crude mouse brain extracts have been investigated in order to identify chemical differences among microtubules which may be important in the control of microtubule assembly or function within cells.

In the present study we have found that a significant percentage of microtubules which polymerize in crude brain extracts in vitro do not depolymerize at 0 °C. We have found that the cold stability is conferred by the incorporation of a factor or factors which we call cold-stabilizing factor (CSF) into microtubules during the process of assembly. Cold stability, at least in part, may be conferred by a low molecular weight substance. We have found that the tubulin isolated from purified cold-stable and cold-labile microtubules is not distinguishable on the basis of gel electrophoresis and isoelectric focusing. Further, we have found that tubulin obtained from completely cold-labile microtubules polymerizes to form cold-stable microtubules when added to tubulin-depleted extracts containing the cold-stabilizing factor. Finally, since the colchicine–tubulin complex prepared from a cold-labile population of microtubules inhibited polymerization of cold-stable microtubules and cold-labile microtubules in identical fashion, we may conclude that tubulin from cold-labile microtubules can copolymerize with cold-stable microtubules. Taken together, these data strongly support the idea that the specific ability to confer cold stability to the microtubules resides in the cold-stabilizing factor and not in the tubulin backbone.

The proportion of cold-stable microtubules formed during polymerization varied considerably depending upon the Ca^{2+} concentration employed during initial homogenization of the brains. Homogenization of the brains at 0 °C in buffer containing 2 mM Ca^{2+} produced extracts that yielded, upon polymerization, 3–6-fold higher proportions of cold-stable microtubules than Ca^{2+}-free homogenization buffers. Further, we observed many short microtubule fragments in brain extracts homogenized at 0 °C in the absence of free Ca^{2+}. However, homogenization of brains in Ca^{2+}-containing buffers solubilized all endogenous brain microtubules. These results suggest that CSF may be a normal component of some fraction of brain microtubules since extraction in Ca^{2+}, which solubilized all of the endogenous brain microtubules, significantly increased the formation of cold-stable microtubules during subsequent polymerization.

The ratio of CSF to total tubulin appears to determine the proportion of cold-stable microtubules which form. The stoichiometry of coassembly of CSF into microtubules was measured by varying the tubulin concentration in crude extract samples (see Figure 10). As the CSF/tubulin ratio increased, the percent cold-stable microtubules formed also increased, to a maximum of approximately 65%. As the CSF/tubulin ratio decreased, the percentage of cold-stable microtubules also decreased, and our results suggest that the effect of CSF can be completely titrated out by increasing polymerization through tubulin addition. This indicates that a critical minimum CSF/tubulin ratio in a microtubule is necessary for cold stability.

The purification of cold-stable microtubules was achieved through two cycles of assembly and disassembly using Ca^{2+} to depolymerize the microtubules and excess EGTA during repolymerization (see Figure 4). These results indicate that CSF remains tightly associated with the assembled microtubules as long as Ca^{2+} is absent and that the integrity and cold-stabilizing activities of CSF are preserved during cycles of polymerization and depolymerization.

However, cold-stable microtubules could not be recycled to yield a pure population of cold-stable microtubules. Rather, a second cycle of assembly, starting with a 100% population of cold-stable microtubules, usually resulted in the formation of about 35% cold-stable microtubules and 65% cold-labile microtubules.

One possible explanation for this loss is that an equilibrium binding reaction may exist between CSF and soluble tubulin or a soluble MAP but that the binding reaction becomes irreversible upon incorporation of a CSF–tubulin or CSF–MAP complex into a microtubule. As noted previously, CSF does not appear to confer cold stability when it is added to preassembled cold-labile microtubules. Further, cold-stable microtubules retain their cold stability for at least 4 h at 0 °C. Thus, it seems reasonable to conclude that CSF does not readily dissociate from a microtubule once it has been incorporated during assembly. However, once disassembled by Ca^{2+}, CSF would dissociate from the tubulin or MAP to a degree determined by the binding constant. This would result in a decrease in the fraction of tubulin or MAP occupied by CSF and, therefore, a reduction in the ratio of cold-stable microtubule formation.

The critical concentrations for polymerization of cold-stable microtubules and cold-labile microtubules were found to be identical (Figure 3). This parameter, which reflects the relationship between the rate constants at the assembly and disassembly ends of the microtubules, is related to

$$1/C_c = K_{eq} = (k_a^{on} + k_d^{on})/(k_a^{off} + k_d^{off})$$

where C_c is the critical concentration, K_{eq} is the equilibrium constant, k_a is the assembly end rate constant, k_d is the disassembly end constant, and off and on refer to the off constants and on constants, respectively. As well as indicating that the CSF incorporates into microtubules during assembly, the similarity of the critical concentrations for the two classes of microtubules places certain restrictions on the possible effects that the CSF might have on the kinetic parameters for assembly and disassembly ends of a microtubule at 30 °C.

For example, one might expect increases in microtubule stability to reflect changes in tubulin off rates from either end of a microtubule. Assuming that k_a^{off} and k_d^{on} are very small at steady state, any decrease in k_d^{off} caused by the presence of the CSF would necessitate a compensating decrease in k_a^{on}. This would have the net effect of slowing the flux rate of tubulin through microtubules containing CSF and might indeed represent one of the effects the CSF would have in vivo.

In summary, differential stability of microtubule populations to various chemical and physical environments must reflect

differences in structures, functional roles, or control properties of the microtubules. Resistance to cold-induced depolymerization is one such difference. The significance of the effects of CSF must await the isolation and identification of the CSF molecule and also, possibly, the identification of more subtle behavioral properties of the microtubules which the CSF may modulate.

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Characterization of Medium Inorganic Phosphate–Water Exchange Catalyzed by Sarcoplasmic Reticulum Vesicles†

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ABSTRACT: Effects of temperature, Ca^{2+}, and ATP on the extent and characteristics of the medium $P_i \rightleftharpoons HOH$ exchange catalyzed by sarcoplasmic reticulum ATPase are reported. Measurements of the patterns of $[^{18}O]P_i$ species formed from highly labeled $[^{18}O]P_i$ show that a single catalytic pathway is involved in the rapid medium $P_i \rightleftharpoons HOH$ exchange with Mg^{2+} present and the much slower exchange with both Mg^{2+} and Ca^{2+} present. A continued high rate of exchange is observed when ATP concentration is increased up to 5 mM even though the amount of phosphoenzyme formed from P_i is much greater at lower ATP concentrations. This result reveals that binding of ATP in some manner causes a pronounced increase in the rate constant for hydrolysis of the phosphoenzyme. During the rapid oxygen exchange in the absence of Ca^{2+} at 10 and 30 °C, the rate of P_i release from the enzyme·P_i complex is about 5–6 times greater than the rate of phosphoenzyme formation. Both P_i release and phosphoenzyme formation are much slower in the presence of Ca^{2+}, with a greater relative tendency for phosphoenzyme formation, particularly at the lower temperature.

Sarcoplasmic reticulum preparations catalyze a rapid exchange of oxygens between P_i and water (medium $P_i \rightleftharpoons HOH$ exchange)[1] in the presence of Mg^{2+} (Kanazawa & Boyer, 1973). This exchange results from the displacement of an oxygen from phosphate by an aspartyl carboxyl group with resultant formation of a phosphoenzyme, E–P (Boyer et al., 1977). Measurement of the rate of exchange P_i oxygens thus allows evaluation of the rate of the reaction step $E{\cdot}P_i \rightleftharpoons E\text{–}P + HOH$. Additional refinements can give the ratio of this exchange rate to the rate of P_i release (Boyer et al., 1977; Boyer & Ariki, 1980). One purpose of this paper is to report the effects of temperature, ATP, and Ca^{2+} on characteristics of the medium $P_i \rightleftharpoons HOH$ exchange and the relevance of these results to the overall reaction mechanism. Of particular interest is the finding that the presence of 5 mM ATP accelerates hydrolysis of the phosphoryl enzyme intermediate.

A second purpose of this paper makes use of the recently developed recognition that the pattern of $[^{18}O]P_i$ species formed from highly labeled $[^{18}O]P_i$ is indicative of whether single or multiple catalytic pathways are involved in the exchange process. Multiple pathways are revealed by heterogeneous distributions of $[^{18}O]P_i$ species containing 0 to 4 ^{18}O atoms per molecule (Sleep et al., 1978). These studies are of additional interest because of the recent demonstration that in the presence of Ca^{2+} and Mg^{2+} two catalytic pathways of cleavage of ATP by sarcoplasmic reticulum become operative at relatively low ATP concentrations (Boyer & Ariki, 1980). Such modulation by ATP concentration of the rate of reversible E–P formation accompanying net ATP cleavage suggests the possibility of an important role of ATP binding in the overall Ca^{2+} transport process. Results reported herein show that although the medium $P_i \rightleftharpoons HOH$ exchange takes place

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[1] Medium $P_i \rightleftharpoons HOH$ exchange occurs when P_i binds, undergoes exchange, and is released to the medium. Intermediate $P_i \rightleftharpoons HOH$ exchange occurs when P_i formed from ATP incorporates more than one oxygen from water prior to release to the medium.

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